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(57) Abstract

Mixed–backbone oligonucleotides POPS blocks have been designed and studied for their target affinity, nuclease stability *in vitro* and *in vivo*, Rnase H–activation properties, and their effect on phosphorothioate–related prolongation of partial thromboplastin time, in an effort to have agents with improved antisense activity with reduced phosphorothioate content.

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MIXED-BACKBONE OLIGONUCLEOTIDES CONTAINING POPS BLOCKS TO OBTAIN REDUCED PHOSPHOROTHIOATE CONTENT

BACKGROUND OF THE INVENTION

Field of the invention

The invention relates to antisense oligonucleotides. In particular, the invention relates to modified antisense oligonucleotides having reduced sulfur content.

Summary of the related art

Mixed-backbone oligonucleotides (MBOs) provide a handle on modulating the pharmacological, pharmacodynamic, and pharmacokinetic profiles of antisense oligonucleotides. MBOs are currently the best choice as second-generation oligonucleotides over PS-oligos. MBOs contain appropriately placed segments of phosphorothioate oligodeoxynucleotide (PS-oligo) and one or more other type of modified oligodeoxynucleotide or oligoribonucleotide. The advantage of MBOs is that, while they retain the advantages of PS-oligo's stability against nuclease and Rnase H activation, the side effects inherent in PS-oligos (immune stimulation, complement activation and prolongation of partial thromboplastin time, etc.) can be minimized, depending on the nature of modified segment incorporated in MBOs. The positioning of the segments of modified oligodeoxynucleotides or oligoribonucleotides in a MBO may strongly affect its desired properties. In end-modified MBOs, a segment of PS-oligo is placed in the center to provide the RNase H activation, and segments of other type of modified oligonucleotide are placed at one or both of the 3'- and 5'- ends to modulate other antisense properties. End-modified MBOs have proved to be more

effective than the PS-oligos as antisense agents and are currently being evaluated in clinical trials as therapeutic agents.

In certain end-modified MBOs, the existence and nature of modifications at the 2'-position of some nucleosides is important in providing increased duplex affinity and stability towards nucleases. The 2'-*O*-methylribonucleoside phosphorothioate and the 2'-*O*-methoxyethoxyribonucleoside phosphodiester are two types of modified nucleotide segments that have been studied most extensively. Incorporation of 2'-*O*-methylribonucleoside in the MBOs can increase the duplex stability with the target RNA. However, for an increase in nuclease stability, phosphorothioate internucleotide linkages are usually required as 2'-*O*-methylribonucleoside phosphodiester segments showed reduced nuclease stability. Incorporation of 2'-*O*-methoxyethoxyribonucleoside also provides an increase in duplex stability, and also demonstrated, *in vitro*, increased nuclease stability even with phosphodiester internucleotide linkages. Both of these types of end-modified MBOs have reduced the PS-oligo-related side effects. Differences in their pharmacokinetic and elimination profiles have been observed, however. The MBOs containing 2'-*O*-methylribonucleoside phosphorothioate show tissue distribution profiles similar to those of PS-oligos following intravenous administration with a significant improvement in stability and retention in tissues; the MBOs containing 2'-*O*-methoxyethoxyribonucleoside phosphodiester showed rapid elimination in urine and disposition in kidneys compared to PS-oligo.

There is a need for additional types of MBOs, which can significantly reduce the PS content without compromising the antisense properties, such as duplex stability, nuclease stability, Rnase H activity, antisense-based biological activity and tissue disposition. Ideally, such MBOs could be obtained by subtle modifications of the best MBOs available to date.

BRIEF SUMMARY OF THE INVENTION

The invention relates to antisense oligonucleotides. In particular, the invention relates to modified antisense oligonucleotides having reduced sulfur content. The invention provides new MBOs, which have significantly reduced PS content without compromising their antisense properties, such as duplex stability, nuclease stability, Rnase H activity, antisense-based biological activity and tissue disposition. These new MBOs are obtained by subtle modifications of the best MBOs available to date.

In a first aspect, the invention provides oligonucleotides containing POPS blocks. POPS blocks are oligonucleotide regions containing alternating nucleoside phosphodiesters (PO) and nucleoside phosphorothioates (PS). In certain preferred embodiments, such nucleoside phosphodiesters and nucleoside phosphorothioates alternate in a one-to-one manner, *i.e.*, PO-PS-PO-PS-PO-PS. In other preferred embodiments, such nucleoside phosphodiesters and nucleoside phosphorothioates alternate in a two-to-one PO to PS manner (PO-PO-PS-PO-PO-PS) or in a two-to-one PS to PO manner (PS-PS-PO-PS-PS-PO). In still other preferred embodiments, such nucleoside phosphodiesters and nucleoside phosphorothioates alternate in a two-to-two manner (PS-PS-PO-PO) or in a three-to-three manner (PS-PS-PS-PO-PO-PO). In yet additional preferred embodiments, the alternation of such nucleoside phosphodiesters and nucleoside phosphorothioates is irregular, provided however, that in such embodiments, a ratio of nucleoside phosphodiesters and nucleoside phosphorothioates of from 1:3 to 3:1 is maintained in at least one POPS block.

In a second aspect, the invention provides hybrid oligonucleotides comprising one or more POPS block. Hybrid oligonucleotides are described in U.S. Patent No. 5,652,355, which is hereby incorporated by reference. Generally, such hybrid oligonucleotides comprise at least one region of deoxyribonucleoside phosphodiesters or phosphorothioates, which is flanked by regions of 2'-O-substituted nucleosides, which may be connected to each other and to the region of deoxyribonucleoside phosphodiesters or phosphorothioates by any type of internucleoside linkage. Thus, in this aspect of the invention, the invention comprises the improvement in a hybrid oligonucleotide of having one or more POPS block as a region of deoxyribonucleoside phosphodiesters or phosphorothioates.

In a third aspect, the invention provides inverted hybrid oligonucleotides comprising one or more POPS block. Inverted hybrid oligonucleotides are described in U.S. Patent No. 5,652,356, which is hereby incorporated by reference. Generally, such hybrid oligonucleotides comprise regions of deoxyribonucleoside phosphodiesters or phosphorothioates, which flank one or more regions of 2'-O-substituted nucleosides, which may be connected to each other and to the region of deoxyribonucleoside phosphodiesters or phosphorothioates by any type of internucleoside linkage. Thus, in this aspect of the invention, the invention comprises the improvement in an inverted hybrid oligonucleotide of having a POPS block as the region of deoxyribonucleoside phosphodiesters or phosphorothioates.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1shows ^{31}P NMR and MALDI-TOF MS spectra of oligo 6. Underlined letters represent deoxynucleosides; plain letters represent 2'-*O*-methylribonucleosides; S and O represent phosphorothioate and phosphodiester linkages, respectively.

Figure 2 shows CGE profiles of comparative stability of oligos **1**, 2 and 6 towards SVPD (0.004 units/50 µl) at 37 °C for 24 hr. Intact oligo **1** was approximately 34%. Peak at 16 min. is of internal standard (PS-oligo 25-mer) added after digestion and before CGE analysis.

Figure 3 shows RNase H hydrolysis pattern of the 5'-^{32}P-labeled RNA phosphodiester 30-mer (5' ACCGCCGCCAGUGAGGCACGCAGCCUU3') in the presence of oligos **1** to 6. Lane – T1, control lane without RNase T1 added; lane +T1, RNase T1 digestion reaction; lane –OH, alkaline hydrolysis reaction; lane-DNA, control RNA lane without any oligo added; lanes oligos **1** to 6, in the presence of oligos **1** to 6 respectively and RNA and RNase H. There was no cleavage in presence of oligos 3, 4 and 5 as they are not substrate for RNase H. Lane oligo X is a treatment in the presence of an oligo which is not included in this disclosure. The structure of the oligos is depicted in Table 1.

Figure 4 shows a comparison of the effects of oligos **1** to 6 on prolongation of aPTT using human blood from healthy volunteer. Each aPTT value is the average of 4 measurements.

Figure 5 shows CGE profiles of extracted samples of oligo **1**(B) and oligo 6(D) from mice plasma at 1 hr post-dosing following IV administration. Panel A and C are control oligo **1** and 6. Peak at 15.5 min. is internal control (PS-oligo 25-mer).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to antisense oligonucleotides. In particular, the invention relates to modified antisense oligonucleotides having reduced sulfur content. The invention provides new MBOs, which have significantly reduced PS content without compromising their antisense properties, such as duplex stability, nuclease stability, Rnase H activity, antisense-based biological activity and tissue disposition. These new MBOs are obtained by subtle modifications of the best MBOs available to date.

The patents and publications cited herein indicate the knowledge in the field and are hereby incorporated by reference in entirety. Any conflict between such patent or publication and the present disclosure shall be resolved in favor of the latter.

In a first aspect, the invention provides oligonucleotides containing POPS blocks. POPS blocks are oligonucleotide regions containing alternating nucleoside phosphodiesters (PO) and nucleoside phosphorothioates (PS). In certain preferred embodiments, such nucleoside phosphodiesters and nucleoside phosphorothioates alternate in a one-to-one manner, *i.e.*, PO-PS-PO-PS-PO-PS. In other preferred embodiments, such nucleoside phosphodiesters and nucleoside phosphorothioates alternate in a two-to-one PO to PS manner (PO-PO-PS-PO-PO-PS) or in a two-to-one PS to PO manner (PS-PS-PO-PS-PS-PO). In still other preferred embodiments, such nucleoside phosphodiesters and nucleoside phosphorothioates alternate in a two-to-two manner (PS-PS-PO-PO) or in a three-to-three manner (PS-PS-PS-PO-PO-PO). In yet additional preferred embodiments, the alternation of such nucleoside phosphodiesters and nucleoside phosphorothioates is irregular, provided however, that in such embodiments, a ratio of nucleoside phosphodiesters and nucleoside phosphorothioates of from 1:3 to 3:1 is maintained in at least one POPS block.

POPS blocks according to the invention comprise from about three to about thirty-five nucleosides, and confer upon, or retain within, an oligonucleotide the ability to activate RNase H. Oligonucleotides containing such POPS blocks also retain important antisense properties, such as duplex stability, nuclease stability, RNase H activity, antisense-based biological activity and tissue disposition.

In a second aspect, the invention provides hybrid oligonucleotides comprising one or more POPS block. Hybrid oligonucleotides are described in U.S. Patent No. 5,652,355, which is hereby incorporated by reference. Generally, such hybrid oligonucleotides comprise at least one region of deoxyribonucleoside phosphodiesters or phosphorothioates, which is flanked by regions of 2'-O-substituted nucleosides, which may be connected to each other and to the region of deoxyribonucleoside phosphodiesters or phosphorothioates by any type of internucleoside linkage. Thus, in this aspect of the invention, the invention comprises the improvement in a hybrid oligonucleotide of having one or more POPS block as a region of deoxyribonucleoside phosphodiesters or phosphorothioates.

In a third aspect, the invention provides inverted hybrid oligonucleotides comprising one or more POPS block. Inverted hybrid oligonucleotides are described in U.S. Patent No. 5,652,356, which is hereby incorporated by reference. Generally, such hybrid oligonucleotides comprise regions of deoxyribonucleoside phosphodiesters or phosphorothioates, which flank one or more regions of 2'-O-substituted nucleosides, which may be connected to each other and to the region of deoxyribonucleoside phosphodiesters or phosphorothioates by any type of internucleoside linkage. Thus, in this aspect of the invention, the invention comprises the improvement in an inverted hybrid oligonucleotide of having a POPS block as the region of deoxyribonucleoside phosphodiesters or phosphorothioates.

In a fourth aspect, the invention provides methods for using oligonucleotides containing one or more POPS blocks to control the expression of specific genes. Such methods comprise administering oligonucleotides according to the invention to cells or to animals, including humans. These methods may be used to assess gene function, or as a therapeutic approach to the treatment of diseases resulting from aberrant gene expression.

Oligonucleotides according to the invention are useful for a variety of purposes. For example, they can be labeled with a reporter group and used as probes in conventional nucleic acid hybridization assays. They can also be used as antisense "probes" of specific gene function by being used to block the expression of a specific

gene in an experimental cell culture or animal system and to evaluate the effect of blocking such specific gene expression. In this use, oligonucleotides according to the invention are preferable to traditional "gene knockout" approaches because they are easier to use and can be used to block specific gene expression at selected stages of development or differentiation. Finally, oligonucleotides according to the invention are useful in the antisense therapeutic approach.

For purposes of the invention, the term "oligonucleotide" includes polymers of two or more deoxyribonucleotide, or any modified nucleoside, including 2'-halo-nucleosides, 2'-O-substituted ribonucleosides, deazanucleosides or any combination thereof. Such monomers may be coupled to each other by any of the numerous known internucleoside linkages. In certain preferred embodiments, these internucleoside linkages may be phosphodiester, phosphotriester, phosphorothioate, or phosphoramidate linkages, or combinations thereof. The term oligonucleotide also encompasses such polymers having chemically modified bases or sugars and/or having additional substituents, including without limitation lipophilic groups, intercalating agents, diamines and adamantane. For purposes of the invention the term "2'-O-substituted" means substitution of the 2' position of the pentose moiety with a halogen (preferably Cl, Br, or F), or an -O-lower alkyl group containing 1-6 saturated or unsaturated carbon atoms, or with an -O-aryl or allyl group having 2-6 carbon atoms, wherein such alkyl, aryl or allyl group may be unsubstituted or may be substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl, or amino groups; or such 2' substitution may be with a hydroxy group (to produce a ribonucleoside), an amino or a halo group, but not with a 2'-H group. Preferably, such oligonucleotides will have from about 12 to about 50 nucleotides, most preferably from about 17 to about 35 nucleotides. Preferably, such oligonucleotides will have a nucleotide sequence that is complementary to a genomic region, a gene, or an RNA transcript thereof. The term complementary means having the ability to hybridize to a genomic region, a gene, or an RNA transcript thereof under physiological conditions. Such hybridization is ordinarily the result of base-specific hydrogen bonding between complementary strands, preferably to form Watson-Crick or Hoogsteen base pairs, although other modes of hydrogen bonding, as well as base stacking can also lead to hybridization. As a practical matter, such hybridization can be

inferred from the observation of specific gene expression inhibition. The gene sequence or RNA transcript sequence to which the modified oligonucleotide sequence is complementary will depend upon the biological effect that is sought to be modified. In some cases, the genomic region, gene, or RNA transcript thereof may be from a virus. Preferred viruses include, without limitation, human immunodeficiency virus (type 1 or 2), influenza virus, herpes simplex virus (type 1 or 2), Epstein-Barr virus, cytomegalovirus, respiratory syncytial virus, influenza virus, hepatitis B virus, hepatitis C virus and papilloma virus. In other cases, the genomic region, gene, or RNA transcript thereof may be from endogenous mammalian (including human) chromosomal DNA. Preferred examples of such genomic regions, genes or RNA transcripts thereof include, without limitation, sequences encoding vascular endothelial growth factor (VEGF), beta amyloid, DNA methyltransferase, protein kinase A, ApoE4 protein, p-glycoprotein, c-MYC protein, BCL-2 protein, protein kinase A and CAPL. In yet other cases, the genomic region, gene, or RNA transcript thereof may be from a eukaryotic or prokaryotic pathogen including, without limitation, Plasmodium falcipa um, Plasmodium malarie, Plasmodium ovale, Schistosoma spp., and Mycobacterium tuberculosis.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature. To carry out the studies, we chose a PS-oligo (18-mer, oligo 1, Table 1) that is complementary to the RIα regulatory subunit of protein kinase A. Oligo 1 has been studied extensively in both *in vitro* and *in vivo* models. In our previous efforts to improve the therapeutic potential of oligo 1, we have studied a MBO (oligo 2), in which four deoxynucleosides from both 3'- and 5'-ends were substituted with 2'-*O*-methylribonucleosides. Oligo 2 has the anti-tumor activities similar to those of oligo 1, but with a significant improvement in pharmacokinetic and toxic profiles observed in mice and rats. Reduction of PS-oligo-related side effects has also been observed. Oligo 2 is presently being evaluated for its therapeutic potential in human clinical trials.

Table 1. Structures of oligos used in this study and their various parameters

Oligo No.	Sequence & Modifications	Tm with RNA (°C)	APTT 50% conc. (μg/ml)
1	5' CsCsGsTsCsCsCsTsCsCsTsCsAsCsTsGsGsC 3'	62.9	37.1
2	5' GsCsGsUsGsCsCsTsCsCsTsCsAsCsUsGsGsC 3'	72.1	46.6
3	5' GsCsGsUsGsCsCsUsCsCsUsCsAsCsUsGsGsC 3'	84.8	81.9
4	5' GoCoGoUoGoCoCoUoCoCoUoCoAoCoUoGoGoC 3"	87.4	>200
5	5' GsCoGsUoGsCoCsUoCsCoUsCoAsCoUsGoGsC 3'	87.2	>200
6	5' GsCoGsUoGsCsCsTsCsCsTsCsAsCoUsGoGsC 3'	77.3	94.1

O – phosphodiester linkage, s – phosphorothioate linkage, underlined – deoxynucleoside, normal – 2" –*O*-methylribonucleoside.

Example 1

Design of oligonucleotides

Based on the design of oligo 2, our approach to further minimize the prolongation of aPTT was to reduce the number of phosphorothioate linkages in oligo 2 without compromising the stability towards nucleases. To carry out the studies, first we designed and prepared some model oligonucleotides (Table 1) to provide insights into the relationship between the nature of the olgonucleotides (nucleoside sugar and phosphate backbone) and its impact on nuclease stability and thermodynamic stability with target RNA, and most importantly, the PS-oligo-related side effects. The oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry on a 15 μmol scale (Expedite 8909, Perceptive Biosystems, MA) or on a 0.5 mmol scale (Pharmacia OligoPilot II Synthesizer). The 2'-*O*-methyl RNA segments with alternative PS/PO internucleotide linkages in oligos 4, 5, and 6 were synthesized by applying the appropriate oxidation reagents in the corresponding synthesis cycles (Beacauge Reagent for PS linkage, and iodine for PO linkage). The oligos were purified by preparative reverse-phase HPLC. The oligo products were characterized by CGE, ^{31}PNMR, and MALDI-TOF MS. These model oligonucleotides included 2'-*O*-methyloligoribonucleoside phosphorothioate (oligo 3), 2' –*O*-methyloligoribonucleoside phosphodiester (oligo 4) and 2'-*O*-methyloligoribonucleoside containing alternative phosphorothioate and phosphodiester linkages (oligo 5).

Example 2

Stability of oligonucleotides

In a study to examine the *in vitro* stability of the oligos towards snake venom phosphodiesterase (SVPD), the following experiments were performed. For each reaction, oligo (0.5 A_{260} units) was suspended in buffer (50 µl) containing Tris (pH 8.5, 30 mM) and $MgCl_2$ (15 mM). To each solution, 0.004 units of SVPD from *crotallus durissus* (Boehringer (Mannheim) was added. The reaction was carried out for 24 hr. at 37 °C. The stability of oligos 1 to 5 is found to be in the order – oligo 3 = oligo 2 = oligo 5 > oligo 1 >> oligo 4. These results suggest that substitution of one phosphorothioate linkage with a phosphodiester in the 2'-*O*-methylribonucleoside at alternative sites does not adversely affect the stability of oligo 5 towards SVPD, compared with that of oligo 3. In a parallel study, it was found that substitution of the phosphorothioate linkage with a phosphodiester linkage in the PS-oligo (oligo 1, Table 1) reduced the modified oligos' stability towards SVPD (data not shown).

Example 3

Stability and duplex formation of a POPS block-containing oligonucleotide

Prompted by the above observation, and the data described later, we designed and prepared a new type of MBO – oligo 6 (Table 1), which contains a PS-oligo segment (nine deoxynucleosides) in the center flanked by five and four 2'-*O*-methylribonucleosides at both the 3' – and 5'-ends containing alternative phosphorothioate and phosphodiester linkages. The structural nature of oligo 6 was confirmed by ^{31}P NMR and MALDI-TOF MS analysis (Figure 1).

In the study to compare the *in vitro* stability of the oligos toward SVPD, nuclease resistance was assessed as described in Example 2. Oligo 6 was found to have stability similar to that of oligo 2, and have greater stability than oligo 1 (Figure 2). This indicated the structural design of oligo 6 had no adverse effects on the oligo's nuclease stability *in vitro*.

In the melting temperature (Tm) study to compare the oligos' binding affinity to the complementary RNA phosphodiester, Tm were recorded using a GBC 920 Spectrophotometer (GBC Scientific Equipment, Victoria, Australia). Oligos were mixed with complementary RNA phosphodiester ((30-mer, 5' ACG GCC GCC AGU GAG GAG GCA CGC AGC CUU 3') in a buffer containing 10 mM Pipes, 1 mM EDTA, and 100 mM NaCl. The Tm values were obtained from the first derivative plots. Oligo 6 showed an increase of 14.4 °C and 5.2 °C in Tm compared with oligo 1 and oligo 2 respectively (Table 1). Compared with oligo 2, the increase of the binding affinity of oligo 6, as demonstrated by the increase of Tm, is due to the substitution of four phosphorothioate linkages with phosphodiester linkages and also an additional 2'-*O*-methylribonucleoside.

Example 4

RNase H activation by a POPS block-containing oligonucleotide

RNase H digestion studies were carried out as follows. For each reaction, the 5' -^{32}P-labeled RNA phosphodiester (30-mer, 0.5 pmol), oligo (5 pmol), and glycogen (50 µmol) were mixed in 12 µl of buffer containing 50 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 200 mM Tris (pH 7.5), and 5% glycerol. Aftere annealing, 0.078 unit of RNase H (Pharmacia) was added to each solution. The mixture were then incubated at 37 °C for 10 min. The reactions were then quenched by adding 20 µl of gel loading dye to each reaction mixture. The resultant samples were analyzed by 20% PAGE and subjected to autoradiography. Oligos 2 and 6 showed to have similar cleavage patterns, which differed from that of oligo 1 due to the flanking 2'-*O*-methylribonucleosides in oligos 2 and 6 [ref. 1] (Figure 3). This study indicated that the MBO design of oligo 6 had no adverse impact on the oligo's ability to cleave the complementary RNA in presence of RNase H.

Example 5

PS-mediated side effects of a POPS block-containing oligonucleotide

Compared with oligo 2, this newly-designed MBO (oligo 6) has less phosphorothioate content, and thus may have less PS-oligo-related side effects. Next, the effects of oligos 1 to 6 on prolongation of aPTT were compared. The study was to see if oligo 6 with a reduced number of phosphorothioate linkages was indeed able to reduce the PS-oligo-related side effects such as prolongation of aPTT. Plasma was obtained from citrated human blood. Serial dilution of the oligos in 0.9% NaCl UPS (saline) were made to provide final concs. of 6.25, 12.5, 25, 50 and 100 µg/ml of oligo in plasma. After addition of the oligo samples, the plasma was incubated at 37 °C for 15 min., with gentle agitation. Plasma exposed to vehicle in the same ratio (v/v) as the oligos, and untreated plasma served as negative controls. The assay was conducted in duplicate, providing at least 2 replication for each tube. The aPTT test was performed by TOXICON (BEDFORD, MA). The results are depicted in Figure 4. All oligos showed concentration-dependent prolongation of aPTT, but with significant differences among the oligos. The clear differences between oligo 1 (PS-oligo) and oligo 3 (2'-*O*-methyloligoribonucleoside phosphorothioate) confirmed our previous observation that phosphorothioate linkage of the oligodeoxynucleoside (PS-oligo) is more effective in prolonging the aPTT than the phosphorothioate linkage of the oligoribonucleoside analogs, including 2'-*O*-methylribonucleoside. As expected, oligos 4 and 5 showed the least prolongation of aPTT, due to the dominant content of the 2'-*O*-methylribonucleoside and the least content of phosphorothioate linkages (Table 1). The concentration required for oligos 4 and 5 to prolong 50% aPTT was more than 200 µg/ml (>35 µM). In general, the prolongation of aPTT in presence of oligos 1 to 6 was in the order – oligo 1 >oligo 2 > oligo 3 > oligo 6 > oligo 4 > oligo 5. To our satisfaction, oligo 6 – the newly-designated MBO in which flanking sequences contain 2'-*O*-methylribonucleosides with alternative phosphorothioate and phosphodiester linkages – showed a significant reduction in its ability to prolong aPTT, compared with oligos 1 and 2. The concentration required to prolong aPTT by 50% for oligos 1, 2, and 6 was 37.1, 46.6 and 94.1µg/ml, respectively (Table 1).

Example 6

In vivo stability of a POPS block-containing oligonucleotide

Prompted by the above *in vitro* results, we extended our study to compare the *in vivo* stability of oligo 6 with that of oligo 1. Oligo 1 and 6 (1 mg) were administered intravenously in mice (female, CD-1, 20-22g) through the tail vein. Following intravenous administration on these two oligos in mice, blood samples were drawn from mice at the post-dosing time points of 30min., 1, 12 and 24 hours. The oligo components were then carefully extracted from the plasma. Part of the oligo samples was analyzed by 20% polyacrylamide gel electrophoresis (PAGE) after the 5'-end labeling with ^{32}P, and part of the oligo samples was subjected to direct CGE analysis (with a UV detector). The PAGE autoradiograph showed presence of bands representing intact length of oligo 6 at much longer time points compared with oligo 1 (data not shown). The increased *in vivo* stability of oligo 6, compared with oligo 1, was also confirmed by the CGE analysis. The CGE profile of oligo 1 showed approximately 55% intact oligo and 45% in degraded form, where as majority of oligo 6 was in intact form (Figure 5). In conclusion, our studies demonstrate that it is possible to optimize the properties of antisense oligos by subtle structural changes in the nucleoside sugar residue and internucleotide, as exemplified by the design of oligo 6. Our preliminary pharmacokinetic study also showed that the tissue disposition profile of oligo 6 is similar to that of oligo 2, which suggests that reduction of the phosphorothioate linkages in oligo 6 does not result in significant changes in tissue deposition (data not shown). Other studies are ongoing to fully exploit the therapeutic potential of oligo 6. Similar design of antisense oligos is applying to other disease models.

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What is claimed is:

1. An improved antisense oligonucleotide, the improvement comprising the presence of one or more POPS block.

2. The improved antisense oligonucleotide according to claim 1, wherein the oligonucleotide is a hybrid oligonucleotide.

3. The improved antisense oligonucleotide according to claim 1, wherein the oligonucleotide is an inverted hybrid oligonucleotide.

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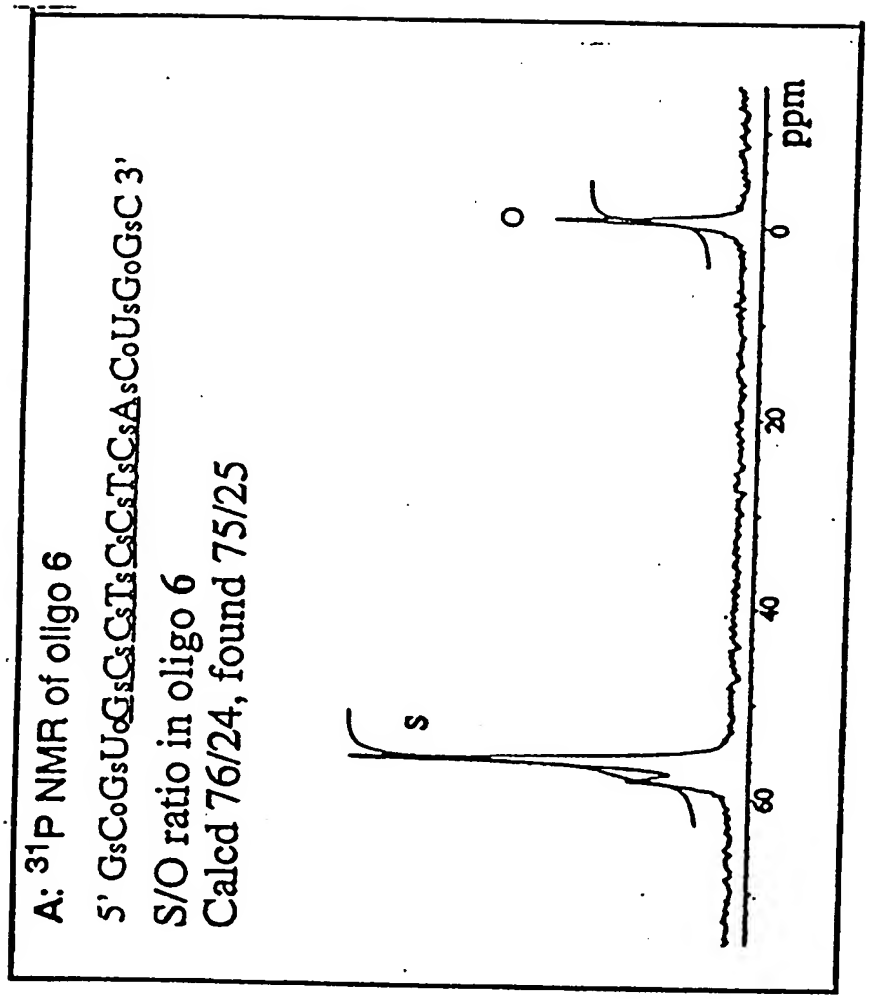
A: 31P NMR of oligo 6
5' GsCoGsUoGsCsCsTsCsCsTsCsAsCoUsGoGsC 3'
S/O ratio in oligo 6
Calcd 76/24, found 75/25
S
O
60
40
20
0
ppm

FIG. 1A

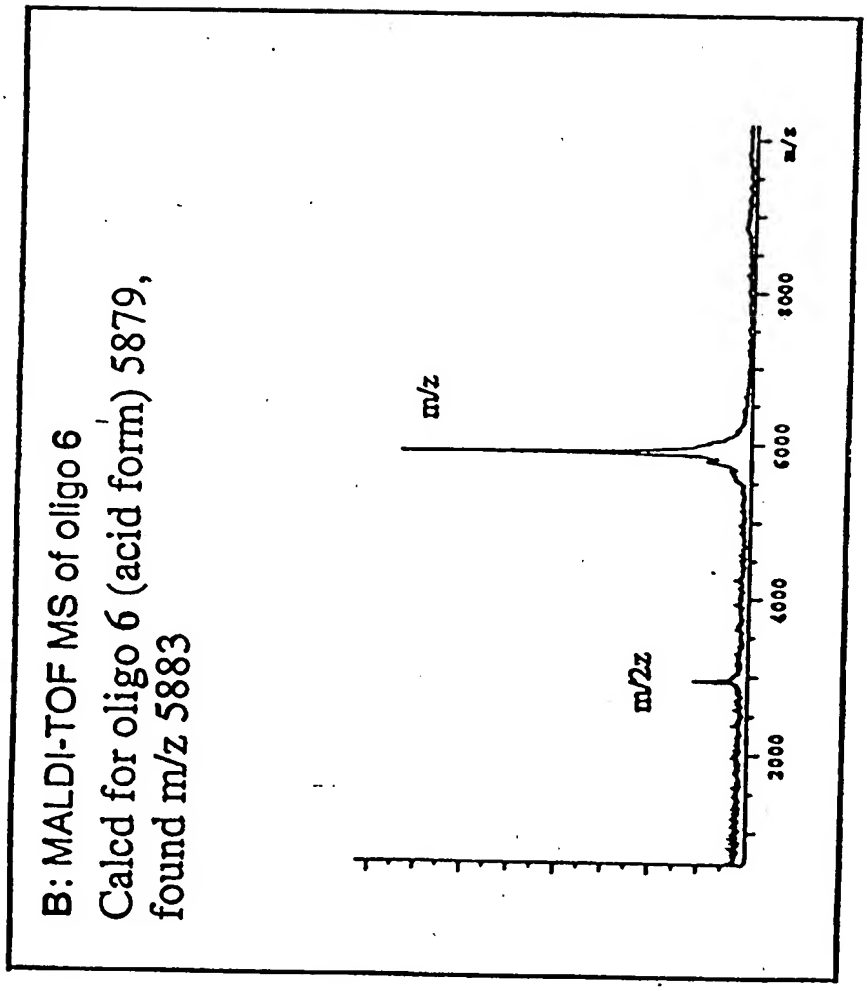
B: MALDI-TOF MS of oligo 6
Calcd for oligo 6 (acid form) 5879,
found m/z 5883
m/z
m/2z
2000
4000
6000
8000
m/z

FIG. 1B

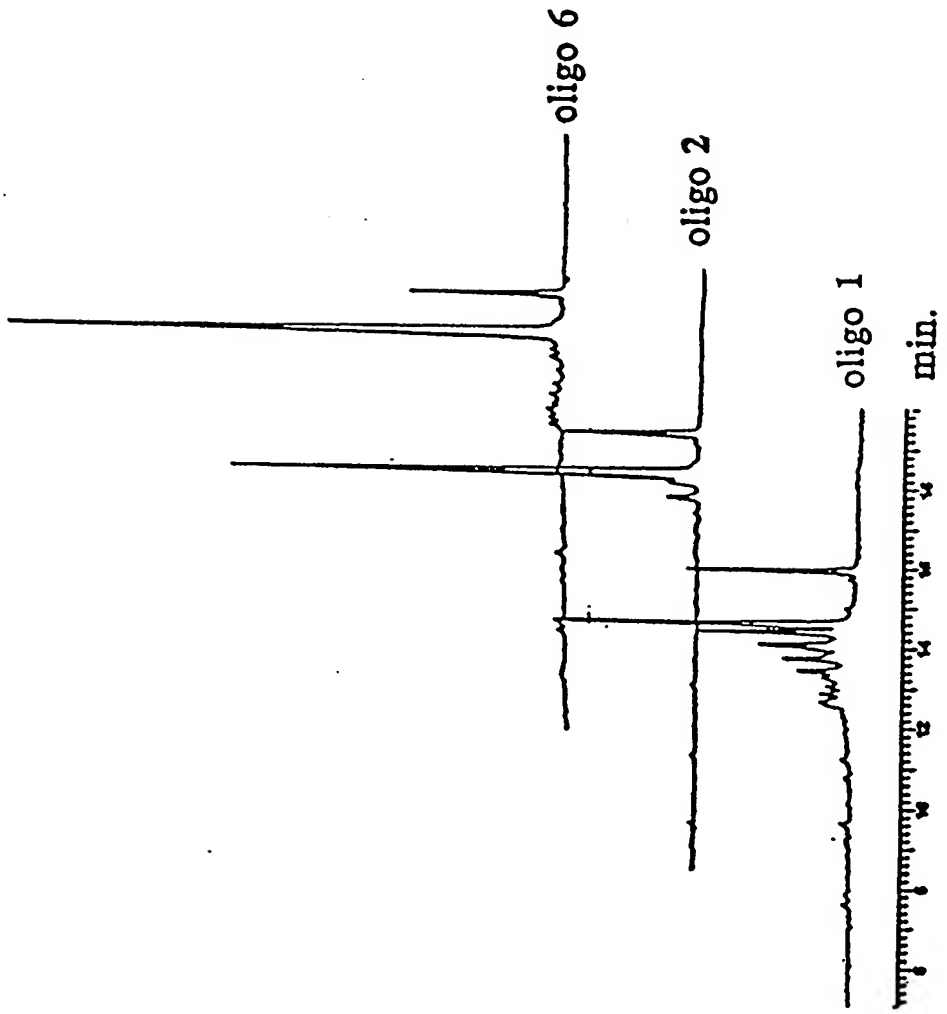

FIG. 2

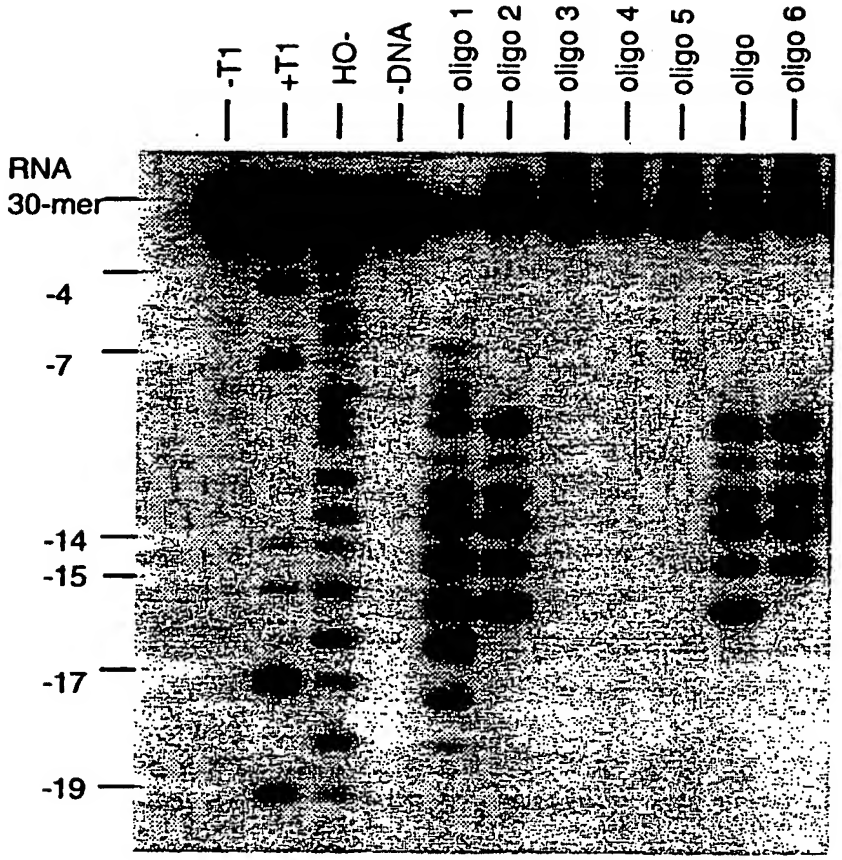
-T1
+T1
HO-
-DNA
oligo 1
oligo 2
oligo 3
oligo 4
oligo 5
oligo
oligo 6
RNA
30-mer
-4
-7
-14
-15
-17
-19

FIG. 3

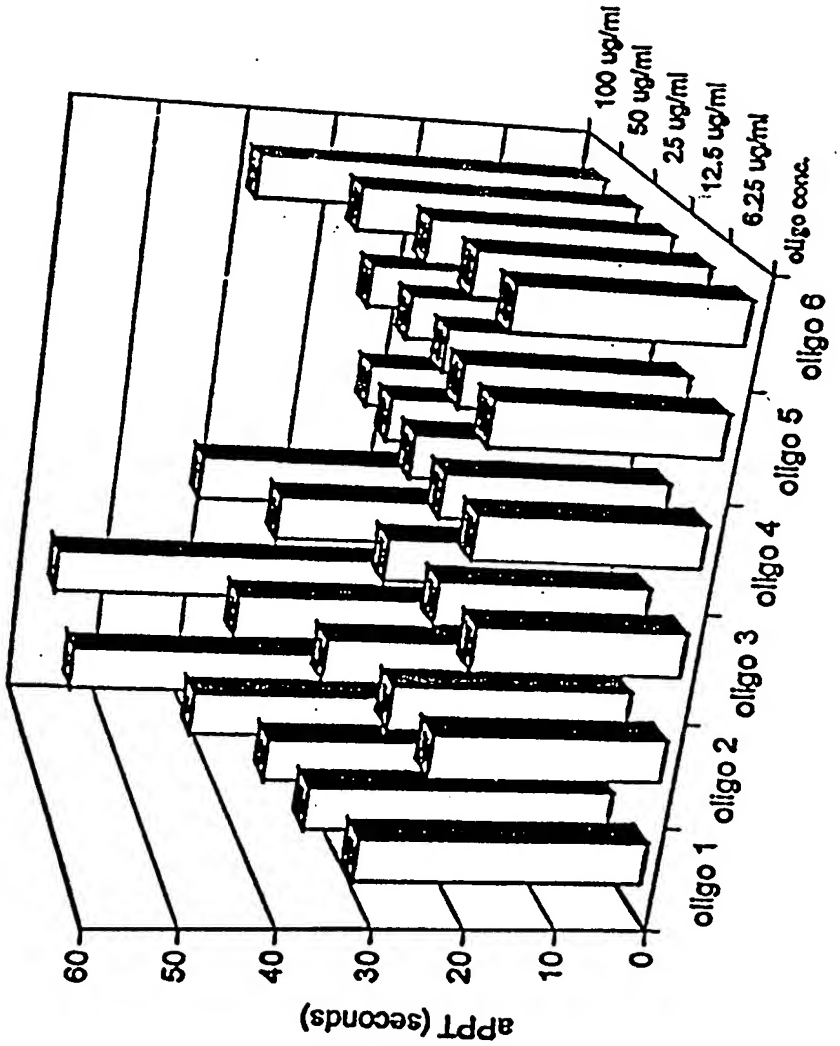
100 ug/ml
50 ug/ml
25 ug/ml
12.5 ug/ml
6.25 ug/ml
oligo conc.
oligo 1
oligo 2
oligo 3
oligo 4
oligo 5
oligo 6
aPPT (seconds)
0
10
20
30
40
50
60

FIG. 4

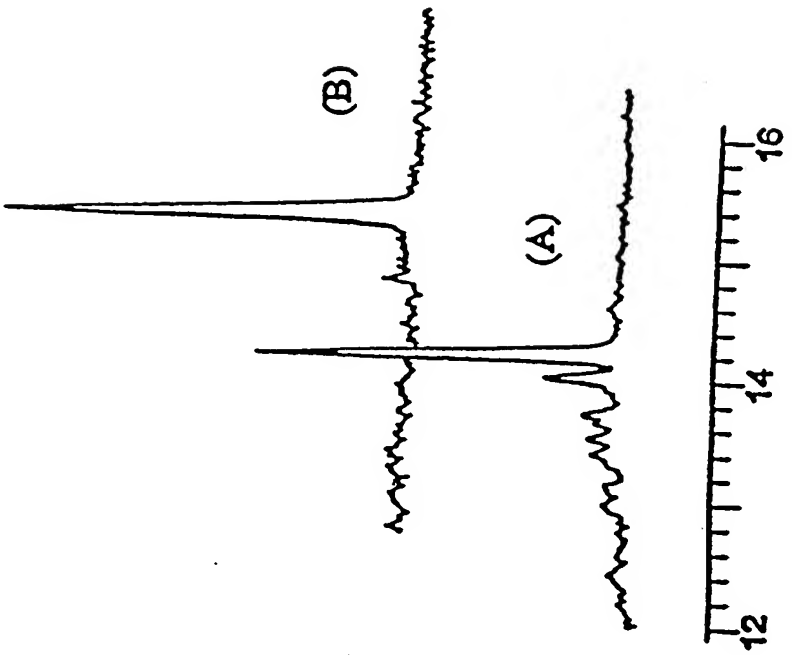

FIG. 5

INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 99/07276

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GHOSH M K ET AL: "Phosphorothioate - phosphodiester oligonucleotide co - polymers: assessment for antisense application." ANTI-CANCER DRUG DESIGN, (1993 FEB) 8 (1) 15-32., XP002110959 the whole document	1
X	WO 95 13834 A (GENTA INC ;ARNOLD LYLE J JR (US); REYNOLDS MARK A (US); GIACHETTI) 26 May 1995 (1995-05-26) page 7, line 32 - page 13, line 4 page 31, line 32 - page 32, line 12 page 74 page 85, line 3,6,7,9 - page 88, line 11 example 43	1-3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 August 1999	12/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

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INTERNATIONAL SEARCH REPORT

Interna. ..al Application No
PCT/US 99/07276

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X	PATIL, SUCHETA V. ET AL: "Syntheses and Properties of Oligothymidylate Analogs Containing Stereoregulated Phosphorothioate and Phosphodiester Linkages in an Alternating Manner." BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, (1994) VOL. 4, NO. 22, PP. 2663-2666., XP002110960 the whole document	1
X	YASWEN P ET AL: "Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells." ANTISENSE RESEARCH AND DEVELOPMENT, (1993 SPRING) 3 (1) 67-77., XP002015214 figure 2D page 73, line 13 - page 74, line 2	1
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P,X	WENQIANG Z ET AL: "Mixed-backbone oligonucleotides as second-generation antisense agents with reduced phosphorothioate-related side effects" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, vol. 8, no. 22, 17 November 1998 (1998-11-17), page 3269-3274 XP004143740 ISSN: 0960-894X the whole document	1,3

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 99/07276

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 49349 A (ECKER DAVID J ;MONIA BRETT P (US); COOK PHILIP DAN (US); FREIER SU) 5 November 1998 (1998-11-05) page 11, line 36 - page 12, line 26 figure 22 -----	1,2

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Information on patent family members

Intern. al Application No
PCT/US 99/07276

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